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Ribose 5'-Phosphate Aminotransferase of Salmonella typhimurium

Gulamnabi Y. Vahora
Utah State University

RIBOSE 5'-PHOSPHATE AMINOTRANSFERASE OF *SALMONELLA TYPHIMURIUM*

by

Gulamnabi Y. Vahora

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of the requirements for the degree

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in

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Approved:

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Major Professor

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Committee Member

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Committee Member

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Dean of Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah

1971

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Finally, I dedicate this work to my parents and to my brothers and sisters, whose incessant love has been a perpetual source of inspiration during my academic career.

Gulamnabi Y. Vahora

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by

Gulamnabi Y. Vahora, Master of Science

Utah State University, 1971

Major Professor: Dr. Carl A. Westby
Department: Bacteriology and Public Health

Ribose-5'-phosphate aminotransferase, an alternate first enzyme in the purine de novo pathway, has been purified about 800-fold from Salmonella typhimurium (pur D-55). The enzyme is distinct from ribosylpyrophosphate-5'-phosphate amidotransferase (E C 2.4.2.14), has an approximate molecular weight of 229,000, and requires ribose-5'-phosphate, ATP and either ammonium ion, carbamyl phosphate or L-glutamine, to synthesize 5'-phosphoribosylamine (PRA). A coupled assay system, employing a S. typhimurium (pur G-310) extract as the coupling-agent source, was used to measure the enzyme. PRA production in this system was inhibited by higher ribose-5'-phosphate concentrations and longer (over 10 minutes) incubation periods. It is proposed that an alternate pathway for the initial step of purine biosynthesis exists in S. typhimurium, namely, the direct conversion of ribose-5'-phosphate and ammonium ion to PRA.

(50 pages)

INTRODUCTION

Free purine and pyrimidine nucleotides are present intracellularly in all forms of life and serve as major components of nucleic acids as well as many co-enzymes. The study of the biosynthesis of these compounds consequently has lead to an understanding of the universality of life.

One approach to the study of purine biosynthesis followed by a goodly number of investigators (1, 5, 8, 24, 34, 36, 60) has been to examine the enzymes of the pathway. Perhaps the first-step enzyme, PP-ribose-P amidotransferase, has received the most attention in this regard because of its dominant role in regulation of the pathway.

This study, on the other hand, reports on an alternate first enzyme of the pathway, ribose-5'-phosphate aminotransferase, catalyzing the reaction:

$$\text{Ribose-5'-phosphate} + NH_4^+ + \text{ATP} \longrightarrow \text{Phosphoribosylamine (PRA)} + \text{ADP} + \text{Pi}$$

The aminotransferase was purified from the cell-free extract of a S. typhimurium mutant (Pur D-55) lacking the second de novo enzyme, (glycinamide ribotide synthetase) GAR synthetase , and various of its physical and biochemical properties were examined. The results of the study confirm the assertions of earlier investigations on the existence of an alternate first

enzyme. The significance of non-enzymatic PRA formation (45) in S. typhimurium now must be re-examined in light of the present study.

REVIEW OF LITERATURE

The De Novo Synthesis of Purine Nucleotides

In the past four decades great numbers of investigators have labored to elucidate the biosynthesis of purine nucleotides in avian (22, 48, 64), tumor (26, 29), plant (32), and microbial systems (1, 12, 17, 34, 43, 51, 56, 60). If it were not for the early emphasis that was given to studies on avian liver, microorganisms might have played a more prominent role in the initial discoveries on purine biosynthesis because purine auxotrophic mutants of bacteria were available early and could have been gainfully employed in Beadle-Tatum (2) types of biosynthetic analyses. As it happened, however, the first studies of purine biosynthesis were in avian liver systems, the most important of these being the works of Schuler and Reindel (53) in 1935, and Edson et al. (11) and Orström et al. (47) in 1936 and 1939, respectively. The particularly important effort of Krebs and coworkers to synthesize purines de novo in pigeon liver slices was the first synthetic study of the pathway at the in vitro level. Hypoxanthine was produced in vitro for the first time in this study, and its synthesis was stimulated by oxaloacetate and L-glutamine (47), suggesting that the nitrogen base was being formed de novo rather than as a result of a breakdown of a pre-formed purine.

The first tissue and cell free system capable of carrying out hypoxanthine synthesis was constructed by Greenberg (18), using pigeon liver extract. His

results indicated that inosinic acid was the first purine compound formed and it was then further catabolized to inosine, hypoxanthine, or uric acid. The work of Greenberg (18, 19) also suggested that purine bases were synthesized from simpler phosphoribosyl derivatives such as ribose-5'-phosphate.

The incorporation of radioactivity into uric acid from labeled CO_2, formate and glycine in in vivo studies (7, 8, 20, 57, 58) indicated that carbon 6 of uric acid was derived from CO_2, carbon 2 and 8 from formate and carbon 4, 5, and nitrogen 7 from, respectively, the carboxyl carbon, the α carbon and the nitrogen atom of glycine (Figure 1). Because of the rapid interchange and loss of N^{15} from N^{15} labeled compounds in the body pools of pigeons, the origin of nitrogen atoms 1, 3, and 9 of uric acid could not be definitely determined by in vivo studies. Levenberg et al. (36) were able to establish the exact origins of these nitrogen atoms, however, by analyzing the degradation products of uric acid and it was ascertained that nitrogen 1 was derived from aspartic acid, and nitrogen atoms 3 and 9 were derived from the amide nitrogen of glutamine.

The other naturally occurring purines were thereafter shown to be synthesized in a similar fashion (5). Figure 1 summarizes the current state of information.

The Formation of β-D-Phosphoribosylamine (PRA)

The initial steps of de novo purine nucleotide synthesis were largely worked out by Goldthwait (13), Goldthwait et al. (14), Levenberg et al. (36),

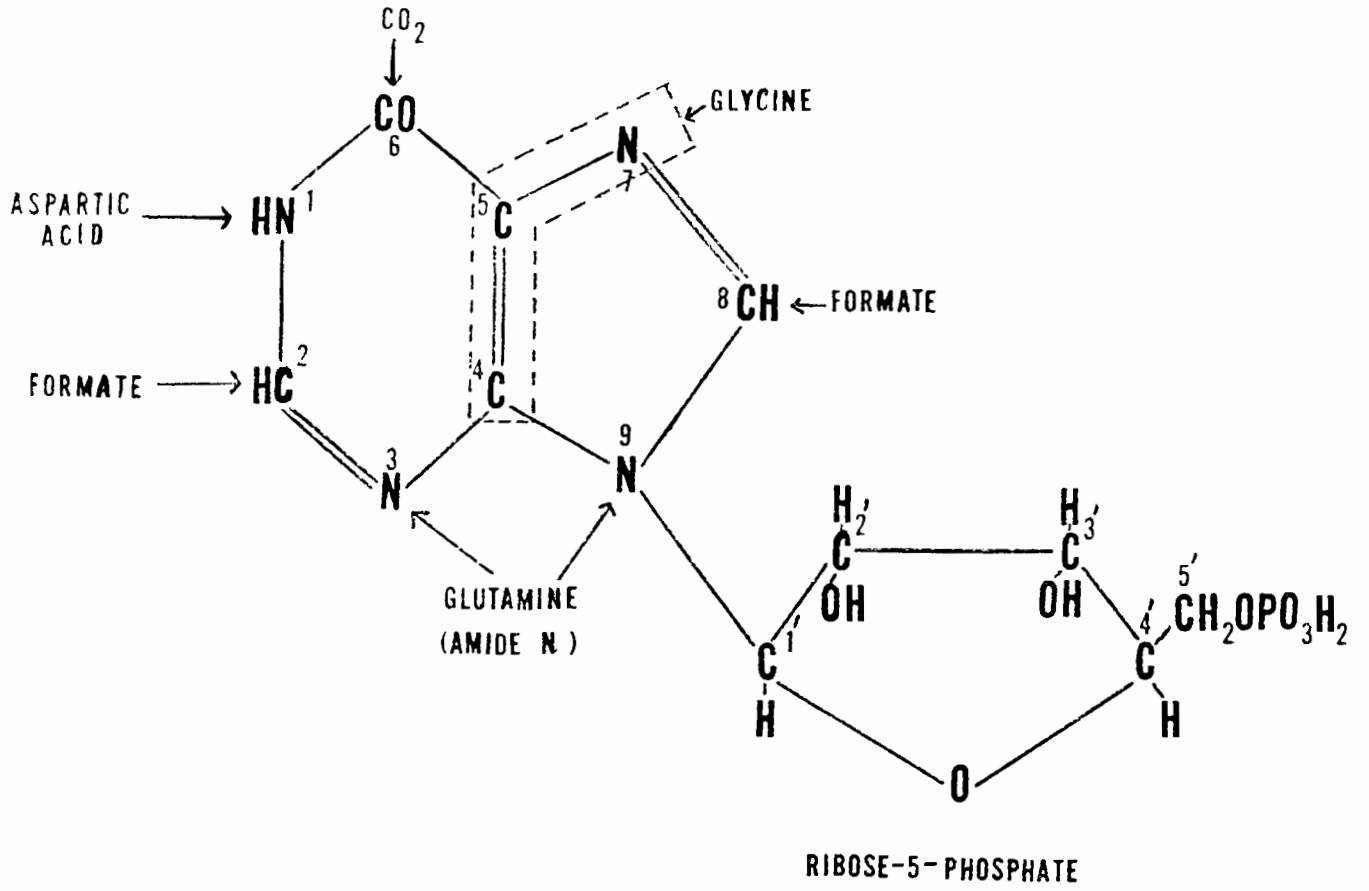


Figure 1. The origin of the carbon and nitrogen atoms of the purine, inosinic acid.

Buchanan and Hartman (6), Hartman and Levenberg (25), Lukens and Flaks (38), and Greenberg (18, 19), in in vitro avian liver studies of the 1950's.

Kornberg et al. (33), and Remy et al. (50), suggested from their studies that the purine biosynthetic pathway was initiated by the formation of 5'-phosphoribosylpyrophosphate (PP-ribose-P) from ribose-5'-phosphate and ATP through the mediation of 5'-phosphoribose-pyrophosphokinase (EC 2.7.6.1). The formation of β-D-phosphoribosylamine (PRA) from PP-ribose-P and L-glutamine was shortly thereafter shown to be the next reaction (22, 26, 49), however, recently an alternate synthesis of PRA from ribose-5'-phosphate and ammonium ion was demonstrated in some of the same systems (29, 32, 34, 45, 48), thus revealing a previously undetected convergence in the de novo pathway. Details of the alternate routes to PRA are indicated in equations 1 and 2.

1. PP-ribose-P + L-glutamine $\xrightarrow[\text{(EC 2·4·2.14)}]{\text{ribosylpyrophosphate 5'-amidotransferase}}$ PRA + PP + L-glutamate

2. Ribose-5'-phosphate + NH_4^+ + ATP $\xrightarrow{\text{ribose-5'-phosphate aminotransferase}}$ PRA + ADP + Pi + H_2O

Ribosylpyrophosphate 5'-Amidotransferase

The PP-ribose-P amidotransferase mediated formation of PRA from PP-ribose-P, L-glutamine, magnesium ion, and ATP is evidenced in plant (32), animal (26, 29, 30, 49), avian (5, 9, 22, 64) and bacterial systems (17, 43, 51, 56). The amide group of L-glutamine seems to serve as the primary

amino source of PRA in pigeon liver (22, 24) and bacteria (17, 43, 51, 56), whereas in plants (32) (wheat embryo) the amide group of L-asparagine serves this role.

The amidotransferase has been purified from pigeon liver (9, 21, 22), ascites tumor (4, 35) and Aerobacter aerogenes (43) approximately 1000-, 20- and 12-fold, respectively. The avian liver enzyme has a molecular weight of about 200,000, contains non-heme iron (9, 21) and has a sedimentation coefficient of 9 S (9 to 11 S for crude enzyme preparation). Rowe and Wyngaarden (52) have reported that the enzyme dissociates rapidly into 100,000 molecular weight subunits and that these, in the presence of thiols, dissociate into 50,000 molecular weight subunits or monomers, which are identical electrophoretically. Each 100,000 molecular weight subunit or dimer, contains 6 non-heme iron atoms, some of which are involved in the catalytic reaction and some of which serve primarily a structural function. The visible absorption spectrum of the enzyme reveals a maximum at 415 mμ, which disappears with the removal of iron from the protein.

The pigeon liver enzyme contains two separate regulatory sites, one for 6-amino and one for 6-hydroxy purine ribonucleotides (22, 23). Mixtures of two non-homologous types of purine ribonucleotides affect a cooperative inhibition on the enzyme (22, 23). It is postulated that the purine ribonucleotides function as allosteric regulators, binding at sites on the enzyme molecule distinct from the active site and inducing a conformational change in the enzyme that renders it catalytically less effective by reducing its substrate affinity (59).

Ribose-5'-Phosphate Aminotransferase

The direct enzymatic synthesis of PRA from ribose-5'-phosphate, ATP and ammonium ion was first suggested by the work of Nierlich and Magasanik in bacterial studies involving A. aerogenes (43). Later the same authors concluded from other evidence that the PRA synthesis they were observing was completely non-enzymic (45), and the ATP requirement was an artifact. Le Gal and co-workers (34) in Escherichia coli studies have since presented opposing evidence, suggesting that PRA is synthesized in vivo from ribose-5'-phosphate and ammonium ion, for the most part, through an enzyme reaction. Other workers also have evidence of such an enzymic synthesis of PRA in other systems (12, 27, 28, 29, 32). Reem (48), for example, has shown that the alternate synthesis of PRA in avian liver occurs only when ribose-5'-phosphate, ATP, magnesium ion, an ammonium ion donor, and an enzyme which is separate and distinct from PP-ribose-P amidotransferase are present. Furthermore, the demonstration of a feed-back sensitivity suggests that the enzymes' role is similar to that of PP-ribose-P amidotransferase in the regulation of purine biosynthesis. The involved enzyme, termed "ribose-5'-phosphate aminotransferase," was purified by Reem approximately 100-fold from duck, chicken, and pigeon liver, and it demonstrated only half the activity of the liver amidotransferase (per mg protein), and was subject to a substrate inhibition by ribose-5'-phosphate.

Gandhi and Westby (12) first reported evidence of the aminotransferase in S. typhimurium. They observed an in vitro synthesis of PRA from ribose-5'-phosphate and NH_4Cl in a system containing a partially purified extract of GAR synthetase. Their work has given impetus to the present study.

The enzymatic synthesis of PRA from ribose-5'-phosphate and NH_4Cl may be of general biological importance because it could provide an alternate means for a typical cell to produce PRA for the de novo synthesis of purines and other end products of the pathway (e.g., thiamine, riboflavin). In Ehrlich ascites cells (29), for example, it appears that the alternate route is indeed operative in vivo and, in fact, is quantitatively more important than the classical route. In gout-ridden and leukemic cells presumptive evidence indicates an increased rate of de novo purine biosynthesis (20, 49, 55). The answer to the larger question, "which route to PRA is more important in other biological systems?," would depend among other things on which enzyme was present in greatest concentration, the substrate Km values, and which enzyme was most subject to feed-back inhibition by the end products. Information on these points is limited, but in regard to the last item both enzymes have been shown to be extremely sensitive to purine nucleotides in various systems (9, 16, 49).

β-D-Phosphoribosylamine (PRA) and Its Conversion to GAR

β-D-phosphoribosylamine, the reaction product of both PP-ribose-P amidotransferase and ribose-5'-phosphate aminotransferase has not yet been isolated in any in vivo or in vitro systems because of its considerable instability. The amine has been prepared chemically, however, by the reaction of ribose-5'-phosphate with liquid ammonia under anhydrous conditions (38), and partially purified as the barium, calcium, or potassium salt. Absolute chemical characterization has been difficult because of the presence of unreacted ribose-5'-phosphate and ammonia in the chemically made preparations and the tendency for

the compound to break down. It has been satisfactorily preserved for periods of several weeks by storage in 0.02 N KOH at low temperatures (38).

In the de novo purine pathway, PRA is converted to glycinamide ribotide (GAR). This conversion was first shown in pigeon liver studies by Goldthwait (13) and Goldthwait et al. (14) and it was subsequently confirmed by Hartman and Buchanan (22), the details are summarized in equation 3.

3. $$\text{PRA} + \text{Glycine} + \text{ATP} \xrightarrow[(\text{EC } 6\cdot 3\cdot 1.3)]{\text{Ribosylamine-5'-phosphate:glycine ligase}} \text{GAR} + \text{ADP} + \text{Pi}$$

The enzyme mediating the reaction, trivially termed GAR synthetase, has been purified from pigeon liver (22), A. aerogenes (45) and S. typhimurium (12). The reaction is reversible and the reaction mechanism has been described in detail by Hartman and Buchanan (23). An equilibrium constant for the reaction could not be obtained because of the apparent destruction of PRA, however, the conversion of GAR to PRA is favored over its synthesis from PRA because of the instability of PRA which pulls the reaction in the direction of PRA.

MATERIALS AND METHODS

Chemicals

D-ribose-5'-phosphate as the sodium salt was obtained from the Matheson Company, glycine-2-^{14}C came from New England Nuclear Corporation, while hemoglobin (Sigma type 1) and beef liver catalase were gifts from Dr. B. F. Burnham (Utah State University). Cellex-D (DEAE cellulose) was purchased from Bio-Rad Laboratories, and the other resin, Dowex-50-H^+ (200 to 400 mesh, 8% cross linkage), was a gift of the Dow Chemical Company. Dowex-50-H^+ was converted to the ammonium ion form by a standard procedure.

Growth of Bacteria

The two purine requiring strains of S. typhimurium employed in the studies were pur D-55 and pur G-310. Both were obtained from the working collection of Dr. J. S. Gots (University of Pennsylvania). Pur D-55 lacks GAR synthetase (63) and is consequently unable to convert PRA to GAR (equation 3 in literature review, p. 10 of this thesis), and pur G-310 lacks a later enzyme (63), formyl-GAR amidotransferase (EC 6.3.5.3) and is consequently unable to convert formyl-GAR to 5'-phosphoribosyl-N-formylglycinamidine. Inocula of the two strains were prepared by placing overnight nutrient broth cultures of each at a 1:100 dilution into vessels containing the E medium (500 ml) of Vogel and Bonner (62) supplemented with glycerol (0.2% W/V), thiamine (1.0 µg/ml) and

monosodium xanthine (2.5 μg/ml) and incubating the flasks at 37°C with bubbling aeration for 10-12 hr.

Sixty liter batches of bacterial cells for use in enzyme purification were prepared by inoculating 5 gallon carboys (4 carboys) containing the supplemented E medium with the 10-12 hr. inocula (1:100 dilution), incubating the vessels at 37°C with bubbling aeration for 12 hr., and harvesting the cells by centrifugation at 3°C. After centrifugation, the cells were washed with 1 liter of 0.005 M sodium phosphate buffer, pH 7.0, and then were frozen overnight in a small amount of the same buffer made 2.0 mM with respect to 2-mercaptoethanol (e.g., 45 gm wet weight cells suspended in 50 ml of buffer).

Preparation of Extracts

Cell-free extracts were prepared by rupturing thawed cells with a Bronwill homogenizer (Type 2876, Bronwill Scientific Inc., Rochester, New York), using the method of Doughty and Mann (10). Good cell breakage coupled with a satisfactory recovery of ribose-5'-phosphate aminotransferase activity was obtained when the homogenization schedule consisted of two, 4000-cycle 2 min. runs.

Crude extract was separated from unbroken cells and debris by centrifugation at 52,000 x g for 20 min. at 0°C. The protein content of crude and purified extracts was determined by the method of Lowry et al. (37).

Purification of Ribose-5'-Phosphate Aminotransferase

A crude, cell-free extract of S. typhimurium, strain pur D-55, prepared as described above, was used in a purification scheme to obtain a ribose-5'-phosphate aminotransferase, free of many other enzymes. The initial purification techniques which were used generally follow the procedures employed by Reem (48) in her purification of an avian liver ribose-5'-phosphate aminotransferase. All steps were carried out at 4°C and all buffers contained 2.0 mM 2-mercaptoethanol unless otherwise stated.

Ammonium sulfate fractionation. Fifty-two milliliters of crude extract prepared as described above were fractionated with 24.5 gm $(NH_4)_2 SO_4$ to produce 70% saturation. The 0-70% fraction lacked aminotransferase activity, however, substantial activity was present in the supernatant (50 ml and 1.85 mg protein per ml) which was dialyzed for 7 hr. against 4.5 liters of 0.005 M sodium phosphate buffer, pH 8.0, to remove the $(NH_4)_2SO_4$ (fraction I).

DEAE cellulose chromatography. A 52 ml portion of the dialyzed extract was placed on a DEAE cellulose column (2.5 x 10 cm) previously equilibrated with 0.005 M sodium phosphate buffer pH 7.0 at 5°C. The column was washed with 20 ml of the same buffer followed by a 600 ml step-wise gradient of decreasing pH and increasing molarity. The gradient was generated with a gradient mixing device, having two side-by-side chambers of equal diameter. The mixing chamber initially contained 300 ml of 0.005 M sodium phosphate buffer pH 7.0 and the reservoir chamber, 300 ml of 0.05 M sodium phosphate buffer pH 6.0. Ten milliliter fractions were collected from the

column after 72 ml of liquid lacking activity had been eluted. When 100 ml more had eluted, the reservoir chamber was made 0.08 M with respect to KCl(14.8 gm). Following this, eluates were collected as before. From each fraction, 0.2 ml and 0.4 ml samples were removed for protein estimation and assay of enzyme activity, respectively. The fractions demonstrating the highest activity (tubes No. 15 and18, second peak fraction) were pooled (Fraction II).

Pressure dialysis. A 40 ml sample from the DEAE cellulose column was added to a pressure dialysis chamber (Amicon Corporation, Diaflo model 200) fitted with a 10,000 molecular weight exclusion ultrafilter (Diaflo PM-10, 62 mm). Nitrogen pressure of 20 psi was applied to the filter, to force the fluid through the filter membrane. After most of the liquid had passed through the filter, the retentate was washed with 20 ml of cooled water made 2.0 mM with respect to 2-mercaptoethanol. The wash was removed by further pressure dialysis. The dialyzates (initial and wash) contained KCl and other salts were discarded. The retentate, in a final volume of 2-3 ml, contained all the proteins of the purified extract. The volume of the retentate was readjusted to approximately 10 ml with cold water containing 2-mercaptoethanol (2.0 mM) and pressure dialysis was repeated with a 50,000 molecular weight ultrafilter (Diaflo PM-50, 62 mm). Most of the proteins, including the aminotransferase, remained again in the retentate and after washing as above, the retentate (6 ml) was subjected a third time to pressure dialysis, in this case with a 100,000 molecular weight ultrafilter (Diaflo XM-100, 62 mm). After the retentate was washed a third time, an examination indicated the aminotransferase was still

present in the retentate which had a volume of 7 ml and contained 160 μg protein/ml. This preparation was called fraction III.

In order to have sufficient purified enzyme for substrate tests, molecular weight analyses and kinetic measurements, the fraction III extracts from two similar purification runs were combined and concentrated by pressure dialysis with a 10,000 molecular weight ultrafilter to a protein concentration of 200 μg/ml.

Assay of Ribose-5'-Phosphate Aminotransferase (for PRA Production)

The aminotransferase catalyzing the ATP dependent conversion of ammonium ion and ribose-5'-phosphate to PRA was assayed indirectly with a coupled system owing to the instability of PRA under physiological conditions. To accomplish the coupling, glycine-2-^{14}C and a partially purified GAR synthetase preparation (0.6-1.0 mg protein of 0-40% $(NH_4)_2 SO_4$ fraction of S. typhimurium pur G-310 extract per assay mixture) were included in the assay system thereby allowing the conversion of any unstable PRA to the stable product GAR, according to equation 3 (in literature review, p. 10 of this thesis). The GAR synthetase preparation was not entirely free from ribose-5'-phosphate aminotransferase, however, an appropriate control was set up in each assay (except in the enzyme dosage experiment) to account for the small amount of PRA produced from the aminotransferase contaminating the coupled system.

A typical incubation mixture contained 30 μmoles tris-HCl buffer (pH 9.0), 1.2 μmoles glycine-2-^{14}C (0.0809-0.112 μc/μmole), 2.7 μmoles $MgCl_2$, 1 μmole ATP, 10 μmoles NH_4Cl, 5 μmoles ribose-5'-phosphate and the two enzyme fractions in a final volume of 0.50 ml.

Control systems contained the same ingredients but were formulated without the second enzyme fraction and thus lacked the aminotransferase. The mixtures (test and control) were incubated at 37°C for 10 min., at which time 0.05 ml of 50% trichloroacetic acid was added to stop any reaction and to precipitate the protein.

GAR-^{14}C and any of the next de novo intermediate, formyl-GAR-^{14}C (mixtures termed "total GAR") were separated from unreacted glycine-2-^{14}C and measured by the method of Westby and Gots (63). Modifications included the use of 0.5 x 7.0 cm Dowex-50 ammonia columns rather than 0.5 x 5.0 cm columns and elution of the "total GAR" from these columns with 8.0 ml of 0.1 N HCl wash rather than with only 3.2 ml. Specific activity was ascertained from the amount of "total GAR" produced according to the method of Gandhi and Westby (12). The "total GAR" was assumed to be equivalent to the PRA.

Determination of Molecular Weight of Ribose-5'-Phosphate Aminotransferase

An estimate of the molecular weight of the ribose-5'-phosphate aminotransferase of S. typhimurium was determined by the sucrose gradient ultracentrifugation method of Noll (46). Sucrose gradients were prepared in 0.05 M

tris-HCl buffer, pH 7.5, made 2.0 mM with respect to 2-mercaptoethanol. Bovine hemoglobin (0.01 ml, 15 mg/ml) or beef liver catalase (0.01 ml containing 13.5 mg protein/ml) or both, as reference standards, were included with the layered enzyme sample (0.08 ml of fraction III containing 200 μg protein/ml) at the time of centrifugation. Tubes were centrifuged on the International Equipment Co., B-60 centrifuge with the SB-283 rotor (Model 488) at 40,000 rpm for 19 hr. at 3-4°C.

To determine the location of the aminotransferase and the catalase, the centrifuged tubes were bottom punctured and six drop samples were collected and assayed for the aminotransferase by the method described above and for catalase by a slight modification of the assay of Weil-Malherbe and Schade (40). The position of hemoglobin in centrifuged tubes was apparent by its color. From the position of the aminotransferase and the references, it was possible to calculate the molecular weight of the aminotransferase using the equations of Martin and Ames (41).

RESULTS

Purification of Ribose-5'-Phosphate Aminotransferase

To ascertain whether the NH_4Cl and ribose-5'-phosphate dependent synthesis of PRA observed by Gandhi and Westby (12) in S. typhimurium was enzymatic or non-enzymatic, an attempt was made to purify from the enterobacterium an enzyme that would catalyze the synthesis. Using the coupled assay described earlier, an enzyme was detected in strain pur D-55 extracts, which brought about the desired synthesis and whose specific activity increased incrementally in extracts undergoing successive purification. A summary of the purification procedure and the extent of purification of the aminotransferase at various steps is given in Table 1.

The second step of purification, DEAE cellulose chromatography, served more than the other steps to purify the ribose-5'-phosphate aminotransferase. The results of the DEAE cellulose chromatography, in terms of the elution of total protein and the enzyme, are indicated in Figure 2. Two peaks of enzyme activity were observed.

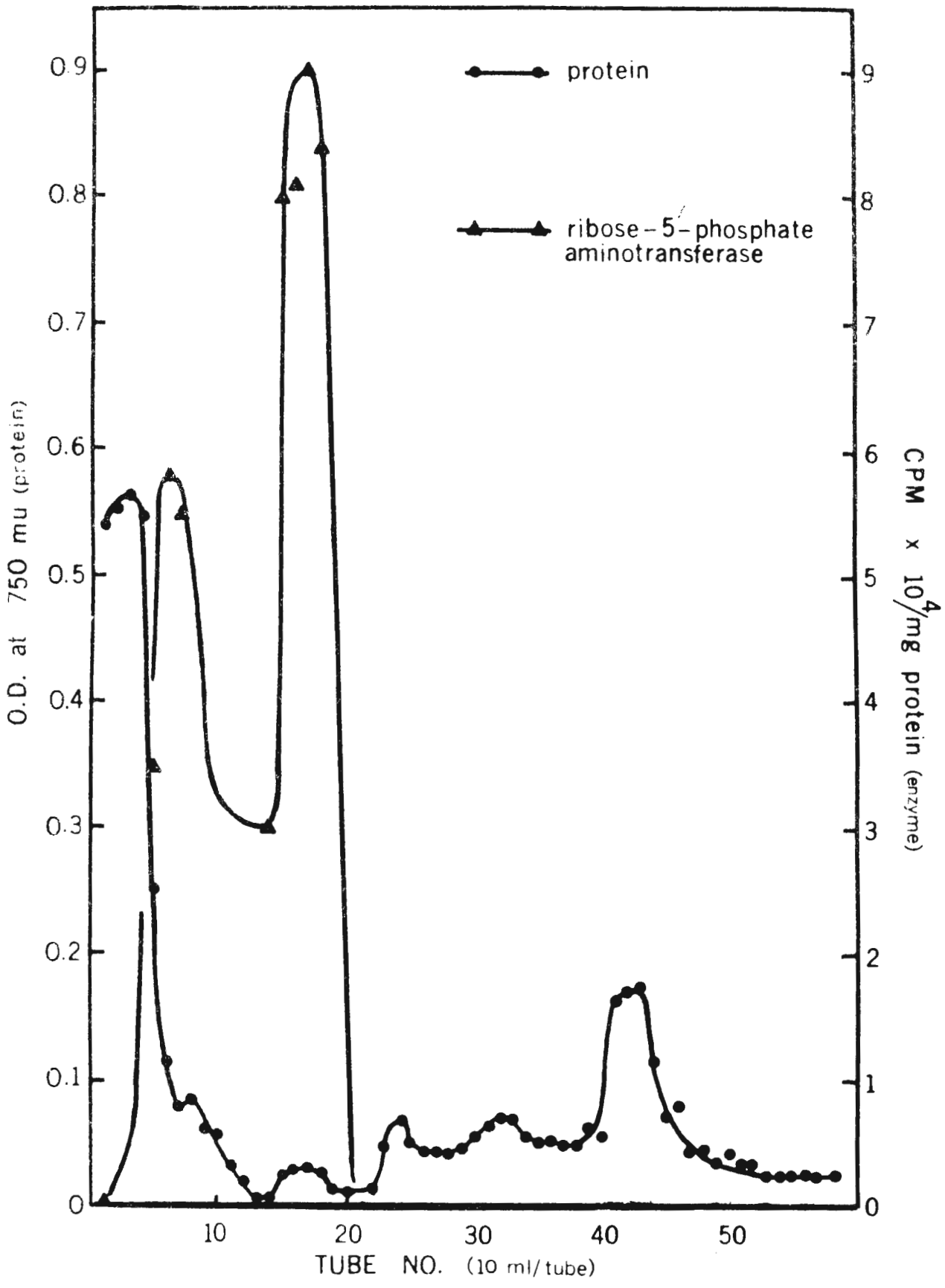
Kinetic Studies

To ascertain the significance of non-enzymatic PRA formation (45) in the assay system used in this study, increasing quantities of ribose-5'-phosphate aminotransferase (from fraction III) were incubated in a series of otherwise

Table 1. Purification of ribose-5'-phosphate aminotransferase from S. typhimurium

Stage and procedure	Volume	Protein	PRA as "total GAR" (nanomoles/mg protein)	Fold purification	Yield %
1. Crude	52.00 ml	8.00 mg/ml	70	1.0	100
2. Ammonium sulfate	50.00 ml	1.85 mg/ml	550	7.92	39.00
3. DEAE cellulose	40.00 ml	230 µg/ml	24.6×10^3	388	19.00
4. Pressure dialysis	10.00 ml	200 µg/ml	55×10^3	792	2.00

Figure 2. Elution of ribose-5'-phosphate aminotransferase from DEAE cellulose column. The procedure is described in "Materials and Methods."


protein
ribose-5-phosphate aminotransferase
O.D. at 750 mu (protein)
CPM x 10⁴/mg protein (enzyme)
TUBE NO. (10 ml/tube)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
2
3
4
5
6
7
8
9
10
20
30
40
50

identical incubation mixtures. The amount of PRA produced in each of these mixtures was proportional to the amount of enzyme present in that system except where saturating levels of the enzyme were present (Figure 3). This suggested that the PRA was, in fact, being produced enzymatically. Extensive dialysis would have removed any substrates from Fraction III, so the increased synthesis of PRA could not be ascribed to anything but large molecules, supposedly protein, present in fraction III. As can be noted in Figure 3, some PRA was formed even in the absence of added aminotransferase. This could reflect either a non-enzymic synthesis of PRA or an enzymic synthesis from small amounts of the aminotransferase contaminating the coupling agent.

The results from another kinetic experiment are indicated in Figure 4. In this test identical incubation mixtures containing a constant level of the aminotransferase, coupling agent and other assay ingredients were incubated for varying periods of time. After 10 min. incubation at 37^{o}C, the amount of PRA that was synthesized surprisingly decreased, and the decrease continued with increasing incubation time. From zero till 10 min., the amount of PRA synthesized was proportional to the time of incubation and it was only after this, that the amount of product decreased. The reason for the decline in PRA production after 10 min. of incubation is unknown, but as a result of the experiment all assays were run for only 10 min.

Molecular Weight Estimation of the Enzyme

Pressure dialysis was employed not only to purify the aminotransferase, but also to obtain a crude estimation of the enzymes molecular weight. On the

Figure 3. Influence of ribose-5'-phosphate aminotransferase concentration on PRA production. The conditions were as described in "Materials and Methods" and fraction III was employed as the source of the ribose-5'-phosphate aminotransferase. At 0 min., only the coupling enzyme was added to the assay system. Each point on the curve represents an average measurement from two identical experiments and each point value actually represents an apparent PRA yield since there was no control subtraction.

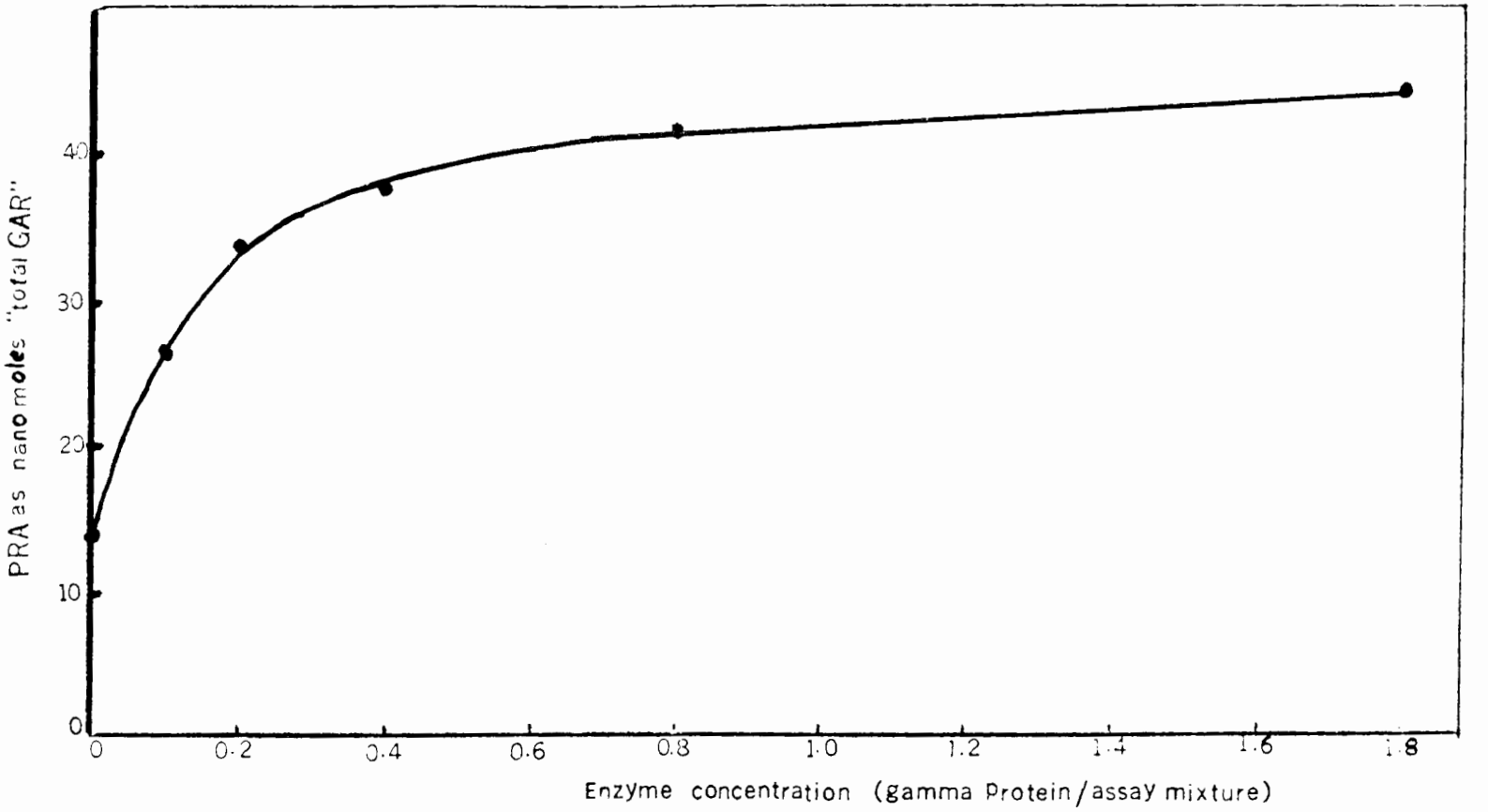
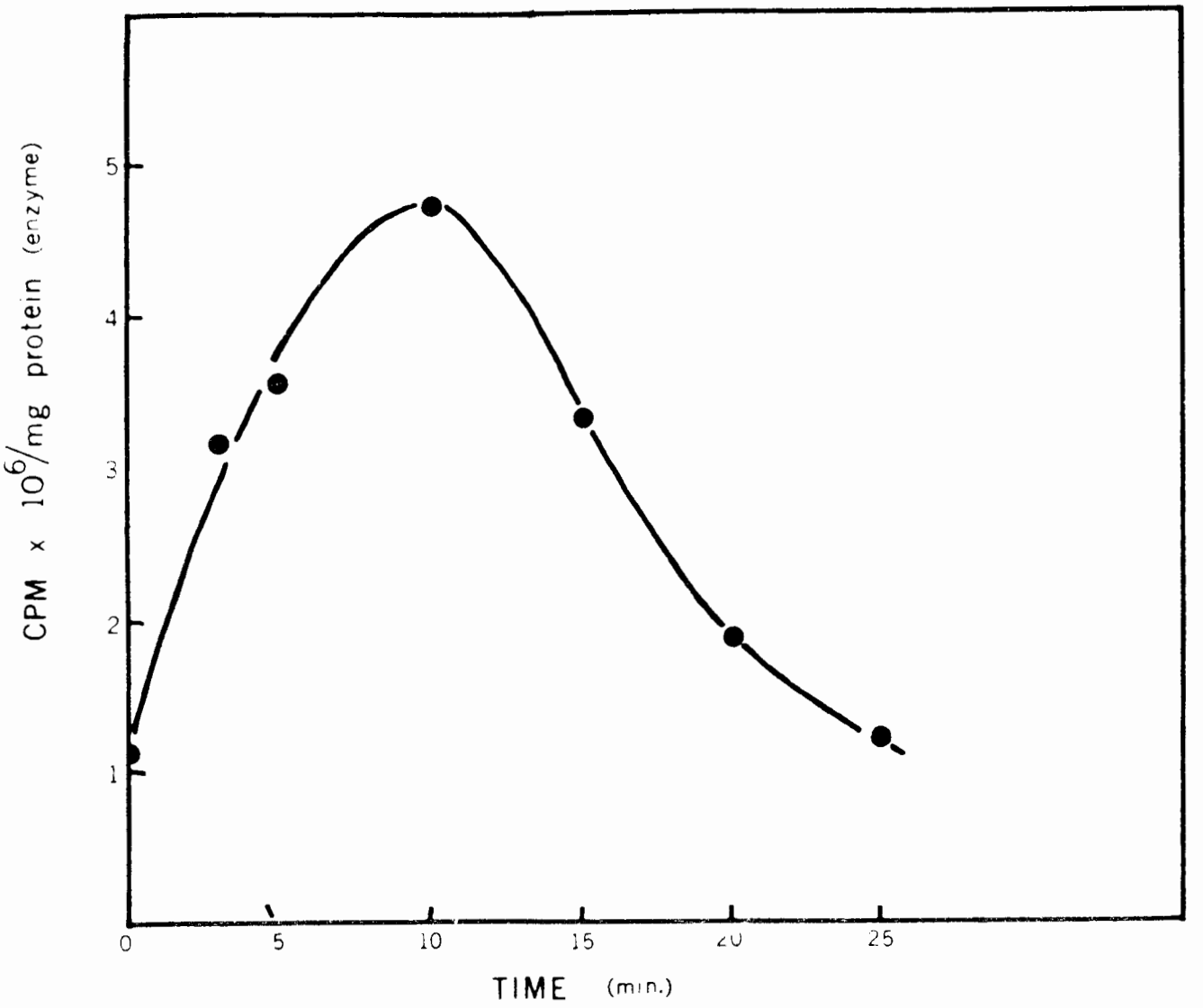

PRA as nanomoles "total GAR"
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
Enzyme concentration (gamma Protein/assay mixture)

Figure 4. Effect of time on PRA production. The conditions were as described in "Materials and Methods" and each incubation system contained 0.6 μg protein of fraction III. Each point on the curve represents an average measurement from two identical experiments.


CPM x 10^6/mg protein (enzyme)
5
4
3
2
1
0
5
10
15
20
25
TIME (min.)

basis of the fact that the enzyme would not pass through a filter pad having a molecular weight exclusion of 100,000, the enzyme was assumed to have a molecular weight greater than this. A nearer approximation of the weight was ascertained by ultracentrifuging a sample of fraction III in a sucrose gradient according to the method of Noll (46). A value of 229,000 (± 12,000) was obtained (Table 2) where beef liver catalase and bovine hemoglobin were used as references and the molecular weight was calculated from equations derived by Martin and Ames (41).

Stability of the Enzyme

No systematic studies on the stability of the aminotransferase were carried out but it was observed that crude, as well as highly purified enzyme preparations, lost only very slight activity after storage at -20°C for 8-10 weeks. Repeated freezing at -20°C and thawing at room temperature had no significant effect on enzyme activity.

Effects of Various Substrates on the Formation of PRA

A series of qualitative and quantitative substrate studies were carried out with the ribose-5'-phosphate aminotransferase of fraction III and the results are indicated in Figure 5 and Table 3. Figure 5 shows the dosage effect of NH_4Cl and ribose-5'-phosphate on PRA formation. This quantitative test was composed of two parts, one of which consisted of a series of identical assay mixtures containing increasing concentrations of ribose-5'-phosphate and a constant concentration of NH_4Cl and the other of which consisted of a series

Table 2. Molecular weight of S. typhimurium ribose-5'-phosphate aminotransferase

Protein	Molecular weight
Bovine hemoglobin	68,000
Beef liver catalase	225,000
Ribose-5'-phosphate aminotransferase	229,000

Note: The procedure is described in "Materials and Methods." The ribose-5'-phosphate aminotransferase molecular weight estimation represents an average determined from two centrifugation runs.

Figure 5. Influence of the concentration of the substrates on PRA production. The conditions were as described in "Materials and Methods" and each incubation mixture contained 0.4 μg protein of fraction III. In the NH_4Cl dosage response test, ribose-5'-phosphate was maintained at 5 μ moles/assay mixture while the NH_4Cl concentration was varied from 0-50 μ moles/assay mixture and in the ribose-5'-phosphate dosage response test, NH_4Cl was maintained at 10 μ moles/assay mixture while the ribose-5'-phosphate was varied from 0-20 μ moles/assay mixture. Each point on each curve represents an average measurement from two identical experiments.

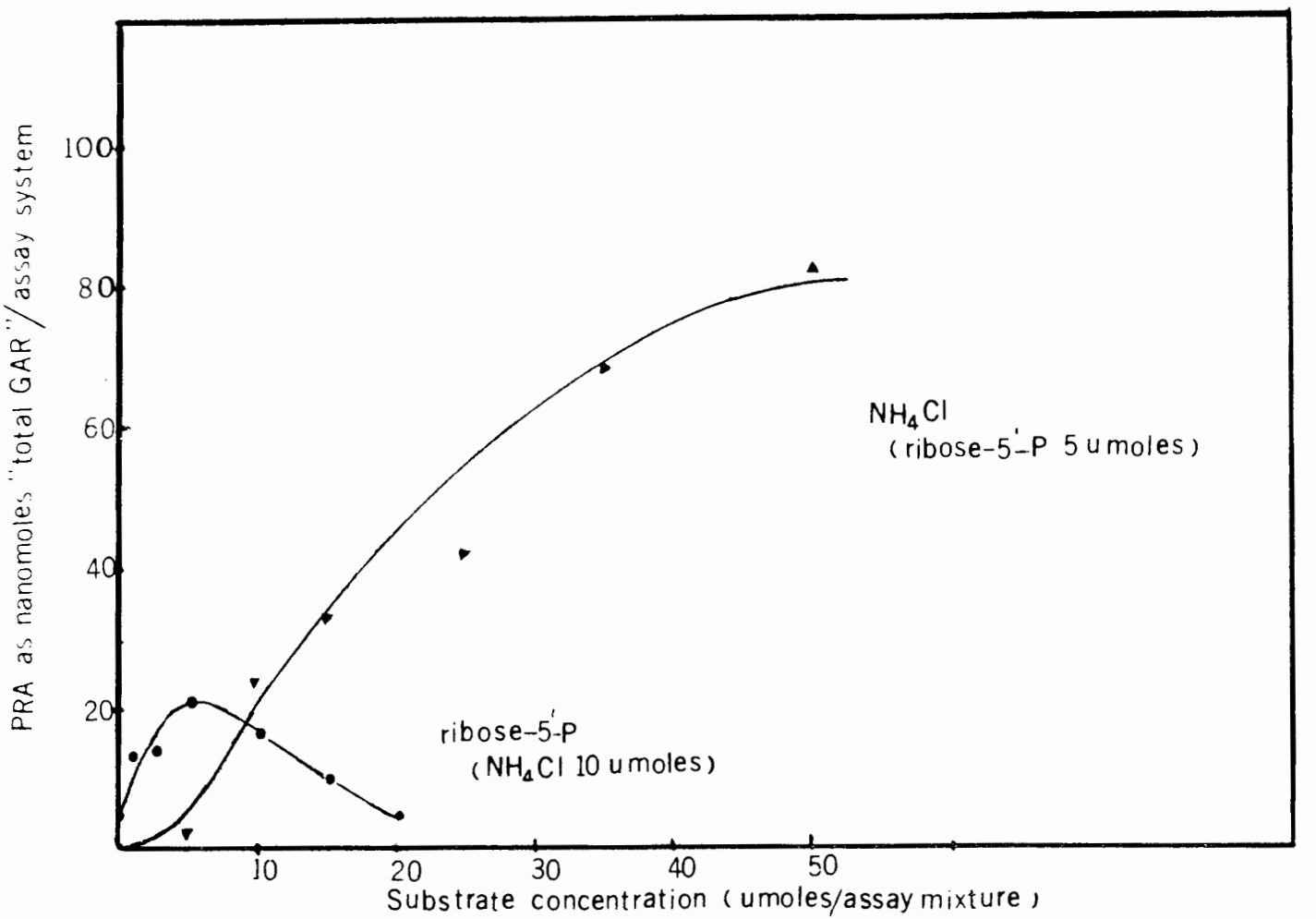

PRA as nanomoles "total GAR"/assay system
100
80
60
40
20
10
20
30
40
50
Substrate concentration (umoles/assay mixture)
NH4Cl
(ribose-5'-P 5 umoles)
ribose-5'-P
(NH4Cl 10 umoles)

Table 3. Effect of various amino donors, PP-ribose-P and methionine sulfoximine on PRA formation

Substrate added	PRA as "total GAR"
	(Nanomoles/assay system)
NH_4Cl	81.1
$(NH_4)_2SO_4$	96.0
Carbamyl-P	114.0
L-Glutamine	153.0
Asparagine	0.0
PP-ribose-P+ L-Glutamine	7.2
Methionine Sulfoximine + NH_4Cl	57.7

Note: The conditions were as described in "Materials and Methods" and each incubation system contained 0.4 μg protein of fraction III. In assay mixtures containing added PP-ribose-P (2 μ moles/assay mixture) and L-glutamine (10 μ moles/assay mixture), there was no added ribose-5'-phosphate or NH_4Cl, all other assay mixtures contained exogenous ribose-5'-phosphate (5 μ moles/assay mixture) and one particular amino donor (10 μ moles/assay mixture). The concentration of methionine sulfoximine was 0.5 μ moles/assay mixture. Each value in the table represents an average measurement from two identical experiments.

containing increasing concentrations of NH_4Cl and a constant concentration of ribose-5'-phosphate. At higher concentrations, ribose-5'-phosphate inhibited PRA formation, whereas NH_4Cl chloride did not demonstrate this inhibitory effect at higher concentrations. For each concentration of ribose-5'-phosphate tested, a control was set up containing that level of the substrate and every other assay ingredient but fraction III, the same was true for each concentration of NH_4Cl tested.

A variety of different nitrogen compounds were tested for their ability to participate as amino donors in PRA synthesis. L-glutamine was found to be the best amino donor followed by carbamyl phosphate, $(NH_4)_2SO_4$ and NH_4Cl. Asparagine did not serve as an amino donor (Table 3).

Because L-glutamine served as such a good amino donor in PRA formation, it seemed possible that the aminotransferase preparation was either contaminated by PP-ribose-P amidotransferase, whose substrate is L-glutamine, or the purified preparation contained the amido-rather than the aminotransferase. To determine, therefore, how much, if any, amidotransferase was present in the purified extract, NH_4Cl and ribose-5'-phosphate were replaced in an incubation mixture by the amidotransferase substrates, L-glutamine and PP-ribose-P. The results show that only about 10% as much PRA as "total GAR" was produced under these circumstances as when ribose-5'-phosphate and NH_4Cl were present. This limited PRA production could have been due to a contamination of fraction III or the coupling agent by low levels of the amidotransferase. Alternatively fraction III and the coupling agent could have been free from contamination and the PRA that was detected in the PP-ribose-P

system could have been produced via the aminotransferase from L-glutamine and low levels of ribose-5'-phosphate (ribose-5'-phosphate was probably present in the commercial PP-ribose-P preparation that was used in the assay). The latter hypothesis would suggest, as the substrate tests just mentioned do, that L-glutamine can substitute for ammonium ion as the amino donor.

To test the adequacy of NH_4Cl as an amino source for the aminotransferase reaction, the glutamine synthetase inhibitor, methionine sulfoximine was employed. Its presence in an incubation system containing NH_4Cl as the sole amino donor was used to determine whether NH_4Cl, itself, was being used as the amino donor or whether the NH_4Cl was first being converted to L-glutamine. If NH_4Cl was being used, the PRA reaction would not be inhibited by methionine sulfoximine, but on the other hand, if the NH_4Cl was first being converted to L-glutamine by the synthetase and the L-glutamine was actually serving as the amino donor, then the reaction would be inhibited by the sulfoximine. The results of such a test are indicated in Table 3. As can be seen, substantial amounts of PRA were still formed in the presence of the sulfoximine. About 70% of the PRA that was produced in the absence of the sulfoximine was still produced in its presence (81.1 nanomoles versus 57.7 nanomoles). This suggests that NH_4Cl, itself, serves as an adequate amino source for the aminotransferase reaction, it also suggests that fraction III primarily contains the amino rather than the amidotransferase, since the latter has a strict requirement for L-glutamine.

DISCUSSION

GAR Production in the Coupled System

In the present study, a purine-requiring mutant (pur-D 55) lacking GAR synthetase (the assay coupling agent) was purposely selected as the source of the aminotransferase rather than the wild type (LT-2) or another mutant. Had a strain been chosen that possessed both the aminotransferase and GAR synthetase (LT-2 or another mutant), then it would have been impossible to use the coupled system to assay for the aminotransferase because the GAR produced in the coupled system in such a case could either have been the result of: (1) the strain's synthetase (utilizing non-enzymatically formed PRA), or (2) the strain's aminotransferase (through conversion of the PRA to GAR by the coupling agent):

1) ribose-5'-phosphate + NH_4Cl $\xrightarrow{\text{non-enzymatically}}$ PRA $\xrightarrow{\text{synthetase}}$ GAR

2) ribose-5'-phosphate + NH_4Cl $\xrightarrow{\text{aminotransferase}}$ PRA $\xrightarrow{\text{synthetase}}$ GAR

By utilizing a pur-D extract it was possible to employ the coupled system with the knowledge that the system was not mistakenly measuring a GAR synthetase activity of the extract.

That the coupled assay was indeed measuring ribose-5'-phosphate aminotransferase activity, and not any non-enzymic PRA formation, was demonstrated in the enzyme dosage-response experiment by the elevated "total GAR" production which occurred in response to increased fraction III

concentrations. The extensively purified enzyme preparation that was employed had been dialyzed several times so that it could not have contained significant amounts of the PRA substrates, and consequently the stimulatory dosage effect could have only been due to high molecular weight material, supposedly enzyme protein. But could this enzymatic material have been PP-ribose-P amidotransferase, which pur-D mutants do possess (63), or was it truly ribose-5'-phosphate aminotransferase? If, in fact, fraction III was comprised of the amidotransferase rather than the aminotransferase, then increasing its dosage in the coupled system could have lead to the increased "total GAR" production that was noted. The aminotransferase substrates, ribose-5'-phosphate and NH_4Cl, could have hypothetically been converted to the amidotransferase substrates, PP-ribose-P and L-glutamine, by contaminant enzymes either in fraction III or the coupling agent. Then any PP-ribose-P amidotransferase present would have converted these secondary substrates to PRA and the latter would have been converted to GAR by the coupling agent. The possibility of fraction III containing the amido- rather than the aminotransferase seems excluded, however, by the fact that when ribose-5'-phosphate and NH_4Cl were substituted by PP-ribose-P and L-glutamine in the coupled system, 90% less "total GAR" was produced than in the normal system. In addition, methionine sulfoximine, an indirect inhibitor of the amidotransferase reaction, only slightly inhibited GAR production in the normal coupled system (containing fraction III and the aminotransferase substrates).

Nierlich and Magasanik (43) observed a non-enzymic formation of PRA from ribose-5'-phosphate and ammonium ion in a coupled system of their design, but concluded that the reaction was quantitatively insignificant in vivo in A. aerogenes. Assuming this to be also true in S. typhimurium, a close relative to A. aerogenes, and based upon the above analysis of the present results, it is concluded that the alternate first step of purine biosynthesis in S. typhimurium proceeds enzymically and in a manner similar to that in wheat germ (32), mammalian tissue (29, 49), E. coli (34) and avian liver (48). In the latter case, the alternate enzyme has been partially purified and completely separated from the L-glutamine dependent amidotransferase. The reaction mediated by the avian enzyme requires ammonium ion, ribose-5'-phosphate and magnesium ion, as does the reaction mediated by the S. typhimurium enzyme. The reaction's overall contribution to purine biosynthesis, however, is still questionable in both systems.

The Ribose-5'-Phosphate Aminotransferase of Various Organisms

Reem (48) working with purified avian-liver ribose-5'-phosphate aminotransferase ascertained that NH_4Cl and $(NH_4)_2SO_4$ served as excellent nitrogen donors for the avian liver enzyme. L-glutamine and asparagine were much less effective and carbamyl-P completely failed to serve as an effective nitrogen donor. The S. typhimurium enzyme shows a somewhat different pattern, as indicated in Table 3. L-glutamine and carbamyl-P were the best donors

followed by $(NH_4)_2SO_4$ and NH_4Cl, asparagine was completely ineffective. Although at variance with the avian liver studies, our results coincide almost exactly with what was found for the aminotransferase of another enteric bacterium, E. coli (34).

The S. typhimurium aminotransferase demonstrated the same response as the avian enzyme to increasing concentrations of the substrates, namely an inhibition to evelated ribose-5'-phosphate levels and the lack of such to elevated NH_4Cl levels. Since ribose-5'-phosphate is not only involved in de novo purine synthesis but also plays a role in the purine salvage pathway (61), pyrimidine synthesis (3, 31, 42) and various other biosynthetic routes (39, 54). It seems reasonable that its usage would be carefully coordinated and regulated. As part of this coordination, there might very likely occur a feed-back inhibition on ribose-5'-phosphate aminotransferase in order to restrict the flow of ribose for de novo purine synthesis.

An 800-fold purification of the S. typhimurium aminotransferase was achieved in the present study as compared to a 100-fold purification achieved in an earlier pigeon liver study by Reem (48). Substrate studies with both the purified bacterial (Table 3) and avian (48) enzyme indicate that ribose-5'-phosphate, and not PP-ribose-P, serves as the pentose acceptor in the amino-transferase reaction.

Ribose-5'-Phosphate Aminotransferase Versus PP-ribose-P Amidotransferase

PRA can be formed in S. typhimurium, not only through the mediation of ribose-5'-phosphate aminotransferase but also through the mediation of PP-ribose-P amidotransferase (15). The latter enzyme requires PP-ribose-P and L-glutamine to form PRA, ribose-5'-phosphate and NH_4Cl will not substitute, and the reaction seems to provide the main route for PRA production (17).

The amidotransferase of pigeon liver has a molecular weight of 200,000 (52) and the enzyme readily dissociates into two 100,000 molecular weight dimers whose monomers have a molecular weight of 50,000. The S. typhimurium aminotransferase has an approximate molecular weight of 229,000. Perhaps, the S. typhimurium ribose-5'-phosphate aminotransferase also dissociates, since in the second step of purification involving DEAE cellulose chromatography, two peaks of enzyme activity were observed. An alternative possibility is that two isozymes of the aminotransferase exist in S. typhimurium.

Feed-back Inhibition

Different workers (15, 16, 44, 48, 49, 56, 65) have demonstrated feed-back inhibition not only of the classical first step, but also of the alternate first step of de novo purine synthesis. Purified preparations of pigeon liver amino-transferase were shown to be inhibited by purines and their derivatives, the nucleotides being more effective than the bases (48). AMP and ATP at concentrations of 4.0 μM inhibited the enzyme reaction 80 and 87 per cent,

respectively, whereas GMP at a concentration of 2.0 mM inhibited the reaction only 30 per cent. ATP was ineffective as an inhibitor and AMP-GMP mixtures exerted no synergistic effect, only an additive one. The situation is somewhat different with regard to pigeon liver amidotransferase, here a wider variety of nucleotides are effective inhibitors at the above concentrations (e.g., AMP, ADP, ATP, GMP, GDP, and IMP) and AMP-GMP mixtures do exert a synergistic inhibitory effect (9, 15, 64). In addition, it was found that the nucleotides acted as competitive inhibitors of the amidotransferase substrate, PP-ribose-P.

In bacterial systems, feed-back inhibition of the amidotransferase has been observed in enzyme extracts from Bacillus subtilis (51, 56), S. typhimurium (15, 17) and A. aerogenes (44). The enzyme from S. typhimurium is inhibited by various nucleotides, of which ADP, GMP, GTP, and AMP are the most effective (15). A similar pattern of end-product inhibition has been observed with the partially purified enzyme from E. coli (15, 34) and it was suggested that the ATP/ADP ratio might be of primary importance.

CONCLUSIONS

1. In vitro kinetic studies indicate that part, if not most, of the β-D-phosphoribosylamine (PRA) synthesized from ribose-5'-phosphate and ammonium ion in S. typhimurium is formed enzymically rather than non-enzymically.
2. The involved enzyme, ribose-5'-phosphate aminotransferase, has been purified about 800-fold from extracts of S. typhimurium (pur D-55) and is distinct from ribosylpyrophosphate 5'-phosphate amidotransferase (EC 2.4.1.14) which is found in the same organism, but which catalyzes the formation of PRA from PP-ribose-P and L-glutamine.
3. The most highly purified preparation of the aminotransferase (fraction III) was either uncontaminated by the amidotransferase or was contaminated by only very small quantities of the enzyme.
4. Alternate amino donors for the aminotransferase reaction, in the order of decreasing effectiveness, include: L-glutamine, carbamyl-phosphate, $(NH_4)_2SO_4$ and NH_4Cl. L-asparagine would not serve as an amino donor.
5. Ammonium ion could be used directly as an amino donor in the aminotransferase reaction without prior conversion to L-glutamine.
6. The aminotransferase was not inhibited by higher concentrations of NH_4Cl, but was drastically inhibited by higher concentrations (above 5 μ moles/ assay mixture) of ribose-5'-phosphate.

7. The aminotransferase has an approximate molecular weight of 229,000 ($\pm$12,000) as determined by ultrafiltration and sucrose gradient ultracentrifugation.

8. A coupled assay, employing a partially purified GAR synthetase extract from another S. typhimurium strain as the source of the coupling agent, was used to measure the enzyme. For unknown reasons, PRA production decreased after 10 min. incubation at 37^{o}C in this system.

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